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Purification and Spectroscopic Characterization of Photosystem II Reaction Center Complexes Isolated with or without Triton X-100[†]

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ABSTRACT: The pigment composition of the isolated photosystem II reaction center complex in its most stable and pure form currently is a matter of considerable debate. In this contribution, we present a new method based on a combination of gel filtration chromatography and diode array detection to analyze the composition of photosystem II reaction center preparations. We show that the method is very sensitive for the detection of contaminants such as the core antenna protein CP47, pigment-free and denatured reaction center proteins, and unbound chlorophyll and pheophytin molecules. We also present a method by which the photosystem II reaction center complex is highly purified without using Triton X-100, and we show that in this preparation the contamination with CP47 is less than 0.1%. The results strongly indicate that the photosystem II reaction center complex in its most stable and pure form binds six chlorophyll *a*, two pheophytin *a*, and two β -carotene molecules and that the main effect of Triton X-100 is the extraction of β -carotene from the complex. Analysis of 4 K absorption and emission spectra indicates that the spectroscopic properties of this preparation are similar to those obtained by a short Triton X-100 treatment. In contrast, preparations obtained by long Triton X-100 treatment show decreased absorption of the shoulder at 684 nm in the 4 K absorption spectrum and an increased number of pigments that trap excitation energy at very low temperatures. We conclude that the 684 nm shoulder in the 4 K absorption spectrum should at least in part be attributed to the primary electron donor of photosystem II.

In photosystem II (PS II)¹ of green plants light energy is used to transport electrons from water to plastoquinone and to contribute to the transmembrane proton gradient. After excitation of one of the pigments of the antenna system of PS II, the excitation energy is rapidly transferred to the primary electron donor P-680, which in its turn transfers in a few picoseconds an electron to a neighboring Pheo *a* molecule (van Grondelle et al., 1994). Further electron transport reactions stabilize this primary charge separation reaction and ultimately result in the formation of reduced plastoquinone and molecular oxygen.

Much knowledge on the most primary photosynthetic processes in PS II has been obtained from the isolated PS II reaction center complex (the D1–D2–cytochrome *b*-559 complex). The most common way to isolate this complex involves the use of the detergent Triton X-100 (Nanba & Satoh, 1987). It is known, however, that the complex is rather unstable in the presence of this detergent. This is most likely due to the formation of singlet oxygen, which can result from oxygen quenching of Chl triplet states (Durrant et al., 1990). Furthermore, Triton X-100 might affect the efficiency of energy transfer (Tang et al., 1991), and it might

also induce a reversible conformational change (Montoya et al., 1994). For these reasons, it is now common practice to exchange Triton X-100 for a mild detergent like *n*-dodecyl β -D-maltoside (DM) after isolation is completed, in order to stabilize the RC when stored or analyzed (Chapman et al., 1988; Seibert et al., 1988). Other methods to reduce the deleterious effects of Triton X-100 involve the application of oxygen-free conditions, the reduction of the time of Triton exposure (van Leeuwen et al., 1991; Kwa et al., 1992a), and the application of a combination of LiClO₄ and DM, which excludes the use Triton X-100 (Ghanotakis et al., 1989; Dekker et al., 1989).

The question of the purity of the PS II RC complex has recently been a matter of considerable debate and so has its pigment composition (Seibert, 1993; van Gorkom, 1995). It has been suggested from spectroscopic (Chang et al., 1994a) and immunological studies (Pueyo et al., 1995) that well-purified PS II RC complexes bind four Chl *a*, two Pheo *a*, and one β -Car molecules and that higher Chl contents arise from contamination with the core antenna complex CP47. This would imply that the photochemical reaction center of PS II binds the same number of pigments as the well-defined reaction center of purple bacteria. A number of other reports, however, have suggested that the isolated PS II RC complex in its most stable and pure form binds six Chl *a*, two Pheo *a*, and two β -Car molecules (Kobayashi et al., 1990; Gounaris et al., 1990; van Leeuwen et al., 1991) and thus that the PS II RC complex has a higher Chl content than the purple bacterial RC. It is very important that the issue of the pigment stoichiometry will be solved, since the number and energies of bound chlorophylls affect the interpretations of functional studies of the PS II reaction center, such as those

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¹ Abbreviations: Car, carotene; Chl, chlorophyll; DM, *n*-dodecyl β -D-maltoside; FWHM, full width at half-maximum; HPLC, high-performance liquid chromatography; Pheo, pheophytin; PS, photosystem; RC, reaction center; T–S, triplet-minus-singlet.

on the primary energy and electron transfer reactions.

In a previous report we analyzed several methods for the determination of the pigment stoichiometry of the PS II RC (Eijkelhoff & Dekker, 1995). It was discussed that previously the Chl to Pheo ratio has sometimes been underestimated to very significant extents (particularly in those studies that promoted the idea of four Chl PS II reaction centers) and that, as yet, all PS II reaction center preparations purified by one of the variations of the "standard" procedure based on incubation with Triton X-100 bind at least six chlorophyll *a* molecules per two pheophytin *a* molecules. Contents of about 6.4 Chl *a* per 2.0 Pheo *a* were found for PS II RC preparations obtained after short or long exposure of CP47–RC complexes to Triton X-100 (Eijkelhoff & Dekker, 1995).

For a proper understanding of the structure and function of the PS II RC complex one needs not only a sensitive method to determine the pigment stoichiometry but also a sensitive method to determine the purity of the isolated PS II RC preparations. In this report, we show that the combination of gel filtration chromatography and diode array detection provides a very powerful tool for the quantitative analysis of the purity of the PS II RC complex. The method makes use of a unique spectroscopic marker (the A_{416}/A_{435} ratio) for the PS II RC complex, which extends the information available from regular chromatograms. We show how to recognize a number of possible contaminants, and we discuss that the sensitivity of this method is superior to that of previously employed immunological techniques. We also show how the method can be used to prepare ultrapure PS II RC particles on an analytical scale without the use of Triton X-100. We present a low-temperature spectroscopic analysis of these ultrapure PS II RC's, show that these preparations give rise to a well-resolved 4 K absorption spectrum, and discuss the nature of the 684 nm absorption feature. Some of the results have been presented at the Xth International Congress on Photosynthesis (Eijkelhoff et al., 1995).

MATERIALS AND METHODS

Gel Filtration. The gel filtration experiments were performed using a Superdex 200 HR 10/30 column (Pharmacia, separation range 10–600 kDa) connected to an FPLC pump (Pharmacia LKB pump P-500) and a diode array detector (Waters 990), which was used in the wavelength range of 280–750 nm with 2 nm resolution. The buffer used as the mobile phase contained 20 mM BisTris, 20 mM NaCl, 10 mM MgCl_2 at pH 6.5 (BT buffer), and 0.03% dodecyl maltoside, and the flow rate was 25 mL/h (for analytical runs) or 40 mL/h (for preparative runs). Samples were filtrated through a 0.45 μm PVDF filter prior to injection and applied to the column using a Pharmacia injection valve fitted with a 200 μL sample loop, due to which the sample volume remained within the recommended 0.8% sample volume range. At a flow rate of 25 mL/h a complete gel filtration run took approximately 40 min. All gel filtration experiments were performed at room temperature in complete darkness.

Preparation of PS II RC Complexes. PS II RC (D1–D2–cytochrome *b*-559) complexes were isolated from CP47–RC preparations from spinach (Dekker et al., 1989) in three

different ways and checked and/or purified using gel filtration. The first two methods are based upon short (~ 15 min) and long (~ 15 h) exposure to Triton X-100 [see Eijkelhoff and Dekker (1995) for details] and yield preparations denoted as RC_{short} and RC_{long}, respectively. The third method (which yields preparations denoted as RC_{DM}) is based on treatment of the CP47–RC complex by a combination of LiClO_4 and dodecyl maltoside as was originally described by Ghanotakis et al. (1989) and Dekker et al. (1989), but with the following modifications. LiClO_4 and DM were added to CP47–RC complexes (at ~ 0.5 mg of Chl/mL) in BT buffer + 1.5% taurine and 0.03% DM to end concentrations of 2 M and 0.5%, respectively, after which the suspension was incubated for 15 min in the dark at 4 °C. The solution was then desalted using a PD10 gel filtration column, after which anion-exchange chromatography (Q-Sepharose) was used as described by Dekker et al. (1989) to separate the RC from CP47 and other contaminants. Most of the CP47 eluted from the column by washing with BT buffer, 0.03% DM, and 20 mM MgSO_4 , whereas the RC was eluted by using the same buffer but with 150 mM MgSO_4 . This RC preparation (denoted from here on as RC_{DM-1}) was further purified by centrifugation on a sucrose density gradient, composed of sucrose solutions in BT buffer + 0.03% DM containing respectively 20–18–16–13–8% (w/v) sucrose. The centrifuge tubes had a volume of 12 mL and could be loaded with a 200–300 μL sample. They were centrifuged overnight using a swing-out rotor (TST41) at 40 000 rpm. The PS II RC fraction was found in the 16% sucrose layer and carefully harvested to prevent contamination with residual CP47–RC complexes in the 18% sucrose layer. The PS II RC fraction was concentrated using a small anion-exchange column (Resource, Pharmacia) to yield the RC_{DM-2} preparation and further purified to RC_{DM-3} by preparative gel filtration chromatography as described above. Only fractions which showed a A_{416}/A_{435} ratio of ≥ 1.16 in the chromatogram were collected. The core antenna protein CP47 was isolated from CP47–RC complexes from spinach as described by Groot et al. (1995).

Pigment Analysis and Spectroscopy. Stoichiometries of Chl *a*, Pheo *a*, and β -Car were determined according to the HPLC and spectroscopic analyses described in detail by Eijkelhoff and Dekker (1995). Low-temperature steady-state absorption spectroscopy was performed on a Cary 219 spectrophotometer, and low-temperature fluorescence spectroscopy was carried out using an imaging CCD camera (Chromex) as detection system and a broad-band 250 W tungsten lamp and 590 nm interference filter as source of excitation light. Temperature-dependent triplet-minus-singlet (T–S) absorbance difference spectra were measured as described by Kwa et al. (1994a), using 665 nm excitation light (4.3 mW/cm²) from a CW dye laser (Coherent 599, DCM dye, spectral bandwidth ~ 2 cm⁻¹) pumped by an Ar⁺ laser (Coherent Innova 310). For all low-temperature measurements the samples were diluted to an optical density of 0.05–0.1 for the fluorescence experiments or ~ 0.5 for the absorbance difference experiments in a buffer containing 20 mM BisTris, 20 mM NaCl, 10 mM MgCl_2 at pH 6.5 (BT buffer), 0.03% dodecyl maltoside, and 70% (w/v) glycerol and placed in a perspex cuvette (1.0 \times 4.0 cm) which was cooled to the desired cryogenic temperature in a He-batch cryostat (Utreks).

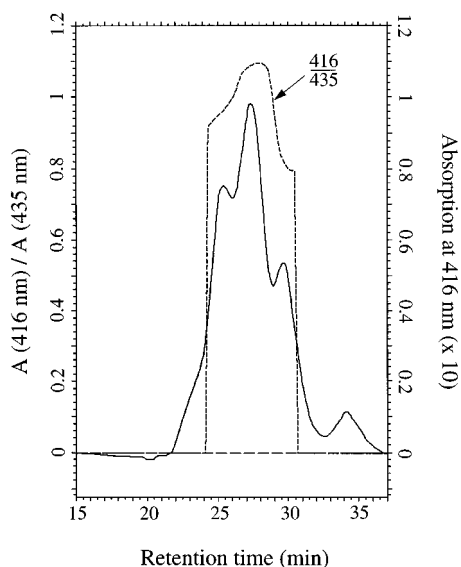


FIGURE 1: Gel filtration chromatogram recorded at 416 nm of a mixture of PS II RC (RC_{short}), CP47-RC, and CP47 complexes. The chromatogram is plotted together with the A_{416}/A_{435} ratio (upper curve), which was calculated with an absorbance threshold of 0.04.

RESULTS

Gel Filtration. In order to assess the composition and purity of isolated PS II RC complexes, we developed a method in which FPLC gel filtration is combined with diode array detection. In Figure 1 the usefulness of this method is demonstrated by presenting a chromatogram of a sample consisting of intentionally mixed CP47-RC, RC, and CP47 complexes. After about 25 min the CP47-RC complex eluted, followed by the PS II RC complex at about 27 min, CP47 at about 30 min, and some free pigment at about 34.5 min. Together with the chromatogram, the A_{416}/A_{435} ratio is plotted (Figure 1, upper curve), because this ratio gives an indication of the Pheo/Chl ratio in the fractions. The A_{416}/A_{435} ratio shows a clear trend as a function of elution time. Starting at 0.90 there is an increase to ~ 1.10 followed by a decrease to 0.80. The trend is in accordance with the values found for the different purified particles (about 0.90–1.20–0.75 for CP47-RC, RC, and CP47, respectively), although the actual value of the ratio is not reached in the case of the RC. This can be explained by the overlap of the RC with the CP47-containing fractions at shorter and longer elution times.

Figure 2 shows the gel filtration chromatogram of a typical RC_{long} preparation. The A_{416}/A_{435} ratio is about 1.20–1.22 through the peak. However, in contrast to the situation in Figure 1, the ratio increases at both sides of the peak, which in the first place suggests that there are no significant CP47-RC and/or CP47 contaminations, since in that case the ratio is expected to decrease. Before the pure RC elutes, larger particles elute from the column with a A_{416}/A_{435} ratio of approximately 1.28. Additional information on the composition of this fraction is obtained by plotting the A_{280}/A_{416} ratio, which gives an indication of the amount of protein per pigment. In the RC peak fraction the ratio is rather straight, with the absorption at 280 nm being about half the absorption at 416 nm. At shorter elution times, however, the ratio increases dramatically, which is most likely caused by contamination with pigment-free, aggregated protein such as disintegrated RC. We note that such type of contamina-

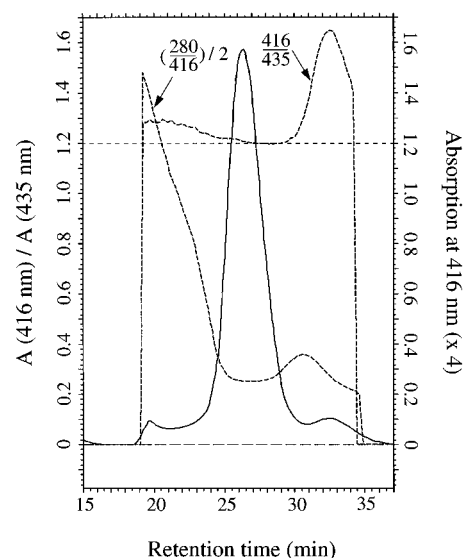


FIGURE 2: Gel filtration chromatogram of a typical RC_{long} preparation, which is a PS II RC complex isolated using a long (~ 15 h) incubation with Triton X-100. The chromatogram is plotted together with the A_{416}/A_{435} and A_{280}/A_{416} ratios, the latter one being divided by two to fit in the picture.

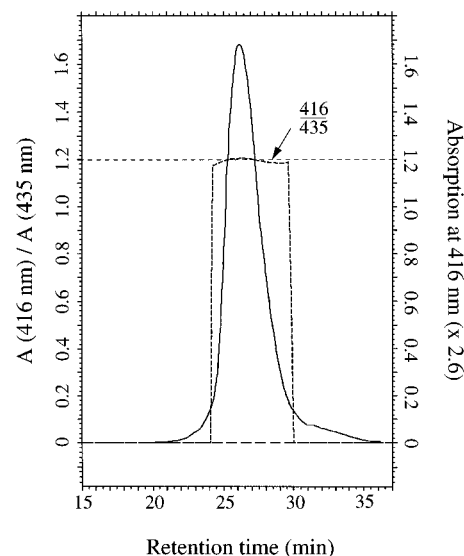


FIGURE 3: Gel filtration chromatogram of a typical RC_{short} preparation, which is a PS II RC complex isolated using a short (~ 15 min) incubation with Triton X-100. The chromatogram is plotted together with the A_{416}/A_{435} ratio.

tion would not have been observed by SDS-PAGE. The higher A_{416}/A_{435} ratio could be caused by extra cytochrome *b*-559 absorption at 416 nm, which suggests that long Triton-treated PS II RC preparations may contain additional, nonfunctional cytochrome *b*-559. At longer elution times, free pigments elute after about 32.5 min. From the A_{416}/A_{435} ratio it can be concluded that free Pheo *a* (which has an extremely high A_{416}/A_{435} ratio) runs slightly faster through the column than free Chl *a*, which implies that the method can also be used to estimate relative amounts of free Chl *a* and free Pheo *a*.

Figure 3 shows the gel filtration chromatogram of a typical RC_{short} preparation. The value of the A_{416}/A_{435} ratio is about 1.20 and constant over the complete chromatogram, suggesting that a very pure RC preparation without significant contaminations was applied to the gel filtration column. The experiment also shows that during the time of the experiment

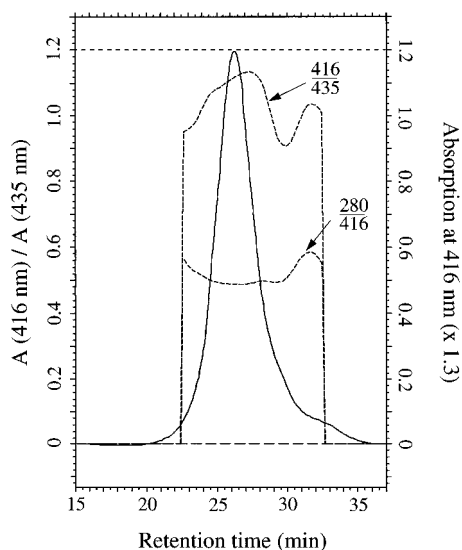


FIGURE 4: Gel filtration chromatogram of a PS II RC_{DM-1} preparation isolated using 2 M LiClO₄ and 0.5% DM (before purification on a sucrose density gradient). The chromatogram is plotted together with the A_{416}/A_{435} and A_{280}/A_{416} ratios.

Table 1: Number of Chlorophyll *a* and β -Carotene Molecules per 2.0 Pheophytin *a* Molecules Determined by HPLC Pigment Analysis

preparation	Chl <i>a</i>	Pheo <i>a</i>	β -Car
RC _{long} ^a	6.3 \pm 0.28	2.0	1.3 \pm 0.20
RC _{short} ^a	6.4 \pm 0.25	2.0	1.6 \pm 0.30
RC _{DM-1}	8.5 \pm 0.50	2.0	2.1 \pm 0.20
RC _{DM-2}	6.6 \pm 0.25	2.0	2.0 \pm 0.10
RC _{DM-3}	6.5 \pm 0.20	2.0	2.0 \pm 0.10

^a Data from Eijkelhoff and Dekker (1995).

(~30 min at room temperature in darkness) the RC does not show any signs of disintegration.

The gel filtration chromatogram of a typical RC_{DM-1} preparation (isolated using a combination of LiClO₄ and DM but without the additional sucrose density gradient purification step) is shown in Figure 4. This preparation was found to contain 8.5 Chl *a* and 2.1 β -Car molecules per 2.0 Pheo *a* molecules (Table 1). Although the chromatogram shows only one peak without apparent shoulders, a closer inspection via the A_{416}/A_{435} ratio reveals shoulders with lower ratios on both sides of the main fraction, suggesting contamination of the RC with both CP47-RC and CP47 complexes. The ratio in the peak itself reaches a value of just 1.13. There is no sign of aggregated protein due to disintegrated RC's because the A_{280}/A_{416} ratio is rather constant at the high molecular mass side of the main peak.

Purification of a PS II RC Complex without Using Triton X-100. The results presented in Figure 4 indicate that RC_{DM-1} complexes can be significantly contaminated with CP47-RC and CP47 complexes. In order to purify the RC from both contaminants, we introduced sucrose density gradient centrifugation as an additional purification step. The pigment ratio of the resulting RC_{DM-2} fraction dropped to 6.6 Chl *a* and 2.0 β -Car per 2.0 Pheo *a* (Table 1), which suggests that at least a large part of the CP47 contamination is removed by this method. In Figure 5 the result of the gradient centrifugation step is shown. The RC fraction is characterized by an A_{416}/A_{435} ratio in the peak of about 1.17, still contains some rather small amounts of CP47 and free

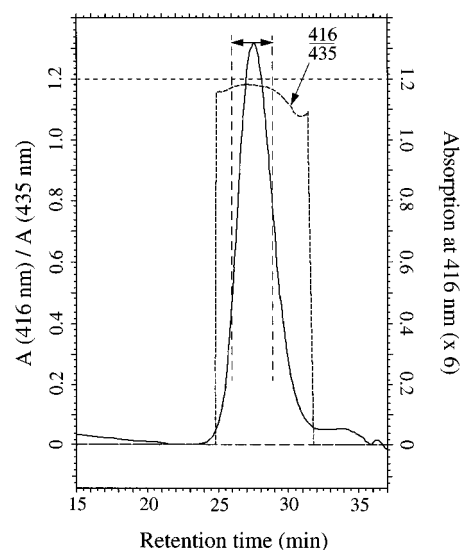


FIGURE 5: Gel filtration chromatogram of a typical RC_{DM-2} preparation, which is a PS II RC complex isolated using 2 M LiClO₄ and 0.5% DM and purified by centrifugation on a sucrose density gradient. The chromatogram is plotted together with the A_{416}/A_{435} ratio. Highly purified RC_{DM-3} preparations were obtained by collecting fractions between 26 and 29 min (indicated by dashed vertical bars and a double arrow).

pigments, but is essentially devoid of CP47-RC. One preparative gel filtration run appeared to be sufficient to remove the residual CP47 and free pigment contaminations (by collecting the fractions that eluted from the column between 26 and 29 min; see Figure 5), after which ultrapure PS II RC_{DM-3} particles have been isolated without significant amounts of CP47, free pigment, free protein, or other contaminants.

The pigment composition of the isolated RC_{DM-3} fraction is shown in Table 1. The Chl content of RC_{DM-3} is only slightly lower than that of RC_{DM-2}, which indicates that the small CP47 contamination in RC_{DM-2} is insignificant within the error and that the gel filtration/diode array detection technique is very sensitive. The Chl/Pheo ratio is essentially the same as in the preparations obtained with Triton X-100, but the β -Car content is significantly higher: 2.0 β -Car per 2.0 Pheo *a* are observed in RC_{DM-3}, whereas we found 1.6 and 1.3 in RC_{short} and RC_{long}, respectively (Eijkelhoff & Dekker, 1995). This suggests that the main effect of Triton X-100 is the extraction of β -Car from the complex. Figure 6 compares the room temperature absorption spectrum of RC_{DM-3} (full line) with those of RC_{short} (dashed line) and RC_{long} (dotted line). The only significant difference between the spectra is found in the β -Car absorption region, which is in line with the results of the pigment analysis. The difference in β -Car content is also reflected in the A_{416}/A_{435} ratio which increases from 1.17 for RC_{DM-3} to 1.25 for RC_{long}.

4 K Absorption Spectra. In Figure 7, the red part of the 4 K absorption spectrum of RC_{DM-3} (full line) is compared with those of RC_{short} (dashed line) and RC_{long} (dotted line). Both RC_{DM-3} and RC_{short} reveal a shoulder at 684 nm, as judged from the second derivative [shown in Figure 7 for RC_{DM-3} and in Kwa et al. (1994a) for RC_{short}]. In the second derivative of the 4 K spectrum of RC_{long}, however, the 684 nm shoulder could not be observed. The results presented in Figure 7 also suggest that the spectrum of RC_{DM-3} shows the highest degree of structure and that of RC_{long} the lowest. This is most easily seen in the region around 675 nm between

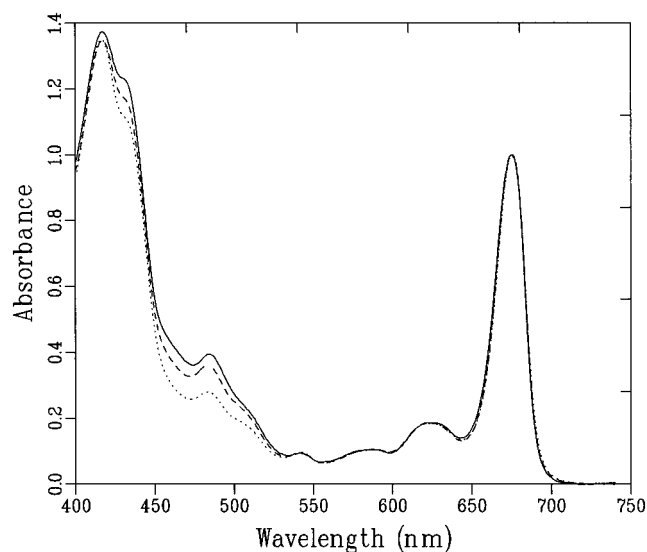


FIGURE 6: Room temperature absorption spectra of RC_{DM-3} (solid line), RC_{short} (dashed line), and RC_{long} (dotted line).

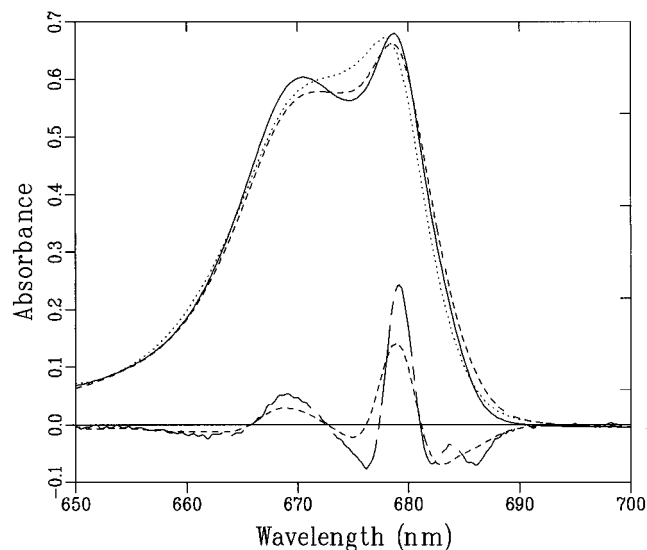


FIGURE 7: 4 K absorption spectra of RC_{DM-3} (solid line), RC_{short} (dashed line), and RC_{long} (dotted line), normalized on their oscillator strengths. The second derivatives of RC_{DM-3} (chain-dashed line) and RC_{long} (dashed line) are shown as well (multiplied with a factor of -3).

the two absorption maxima, which shows a very pronounced valley in the case of RC_{DM-3} , a very small valley in the case of RC_{short} , and no valley at all in the case of RC_{long} .

In order to get more information on the spectral fine structure of the 4 K absorption spectra, we deconvoluted the spectra into Gaussian components. We are well aware of the limitations of this procedure, since a unique solution is seldomly found and since there is no good evidence that the absorption bands have Gaussian shapes. Nevertheless, analysis of the spectral window of 660–690 nm yielded consistent results of all three spectra with five spectral components (Table 2). More components will result in even better fits [see, e.g., Cattanea et al. (1995)] but appeared not necessary to extract the basic features. The most important difference between RC_{DM-3} and RC_{short} is the slightly larger bandwidth of the components peaking near 683 and 679 nm; the other features are virtually the same. This suggests that relatively small changes in the bandwidths of the absorption bands might be responsible for the observed spectral differ-

Table 2: Results of Fits with Gaussian Bands of 4 K Absorption Spectra between 660 and 690 nm

RC_{DM-3}					
peak wavelength (nm)	683.0 ^a	679.2	675.4	670.9	660.0
FWHM (nm)	4.6	4.8	4.0	12.3	22.0
height ^b	0.1426	0.4690	0.0795	0.5342	0.1300
area (%) ^c	6.7	23.0	3.3	67.1	
RC_{short}					
peak wavelength (nm)	683.0 ^a	679.1	674.6	671.3	663.2
FWHM (nm)	6.0	5.6	3.9	13.0	24.4
height ^b	0.1133	0.3950	0.0413	0.4682	0.1371
area (%) ^c	7.3	23.9	3.1	65.7	
RC_{long}					
peak wavelength (nm)	683.0 ^a	678.9	675.0	671.6	663.8
FWHM (nm)	5.2	5.5	4.7	13.3	23.9
height ^b	0.0574	0.3676	0.0634	0.4616	0.1583
area (%) ^c	3.4	23.1	3.4	70.1	

^a The wavelength of the red-most absorption band was frozen at the same wavelength in the three preparations in order to stress the differences between the preparations. Inclusion of this wavelength as a free fit parameter usually resulted in slightly different fits which, however, did not differ significantly in fit quality. ^b The three absorption spectra have been normalized on their oscillator strengths and on the pigment contents determined in Table 1. ^c The fifth band is disregarded in this percentage because it largely represents vibrational transitions of the four other bands.

ences between RC_{DM-3} and RC_{short} and that a short Triton X-100 treatment does not induce significant changes in the structural integrity of the pigment–protein complex. The most important difference between RC_{short} and RC_{long} seems to be the smaller amplitude of the 683 nm absorption band in RC_{long} , which suggests that this absorption band is more labile than the main band peaking near 679 nm.

Temperature-Dependent Fluorescence. The 4 K emission spectrum of the purified RC_{DM-3} complex (not shown) appeared to be virtually identical to the 4 K spectrum of RC_{short} (Groot et al., 1994; Kwa et al., 1994b) and is characterized by a narrow band (FWHM ~ 8.5 nm) peaking at 683.6 nm. By measuring the fluorescence quantum yield as a function of the temperature, the interactions between the different pigment pools can be studied. At 4 K we expect that the excitation energy will be trapped on the chlorophyll with the red-most absorption. If this chlorophyll is not part of the primary electron donor P-680, the excited state cannot escape via charge separation and will to a much larger extent decay via fluorescence [see, e.g., Groot et al. (1994)]. The yield of the fluorescence as a function of temperature will therefore provide an indication of whether or not such trapping chlorophylls will be present and, in addition, will provide information on the energetics of the uphill energy transfer to P-680.

Figure 8 shows that the temperature dependence of the fluorescence yield of RC_{DM-3} (open triangles) is very similar to that observed earlier in RC_{short} [open circles; data from Groot et al. (1994)]. Qualitatively, the decrease of the emission yield upon lowering the temperature from about 160 to about 80 K is explained by the shift of the equilibrium between the radical pair and the excited state toward the radical pair, while the decrease upon raising the temperature above 160 K is explained by a temperature dependence of the excited state decay via internal conversion (Groot et al., 1995) and the increase upon lowering the temperature from 60 to 4 K by increased contributions of trap pigments (Groot et al., 1994). Combined with the biochemical evidence reported above, this indicates that the trap of excitation

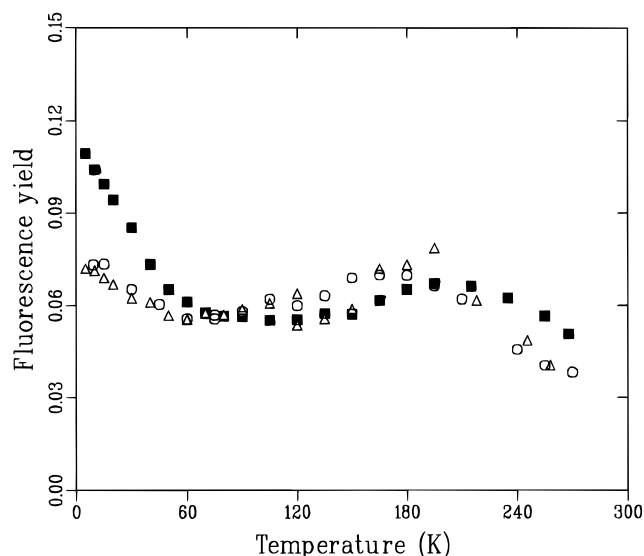


FIGURE 8: Fluorescence quantum yield as a function of temperature of RC_{DM-3} (open triangles), RC_{short} [open circles; data from Groot et al. (1994)], and RC_{long} (closed squares), obtained by integrating the emission spectra from 650 to 700 nm as described in detail by Groot et al. (1994).

energy in the PS II RC is not due to contaminating Chl-protein complexes but really is a feature of the pure complex. It furthermore indicates that the overall energetics of the P-680 and trap Chl's of the PS II RC system are similar in both preparations, which is in line with the results from the Gaussian decomposition of the 4 K absorption spectrum.

In RC_{long} , however, the relative fluorescence yield is larger at 4 K than of RC_{DM-3} and RC_{short} (Figure 8, closed squares), which suggests that in this preparation there is a higher probability that excitations get trapped on red antenna chlorophylls at very low temperatures. These results suggest that there is an inverse relationship between the amplitude of the 684 nm absorption band and the amount of centers in which the excitation is trapped on red antenna chlorophylls at very low temperatures.

Triplet-minus-Singlet Absorbance Difference Spectra. We used the ultrapure RC_{DM-3} preparations described above to record the temperature dependence of the (nonselectively) triplet-minus-singlet (T-S) absorbance difference spectrum (also called the non-line-narrowed transient hole-burning spectrum). Such a dependence was recently also recorded by Chang et al. (1995) on a preparation that according to the authors was enriched in 684 nm absorbing chlorophylls [note that the Chl/Pheo ratio may have been considerably larger than the ratio mentioned by Chang et al., as was already noted by the authors; see Eijkelhoff and Dekker (1995)].

Figure 9 shows T-S spectra of RC_{DM-3} obtained by nonselective excitation (665 nm) at 4, 15, 30, and 56 K. The spectra are characterized by a relatively broad bleaching peak near 680 nm and a shoulder at 684 nm and are very similar to the T-S spectra of Triton-derived preparations (van Kan et al., 1990; Kwa et al., 1994a). We note that no decrease of signal could be detected between successive measurements at each of the temperatures and that the spectral changes are completely reversible.

In contrast to the results of Chang et al. (1995) the contribution of the 684 nm shoulder in the T-S spectrum of RC_{DM-3} undergoes no significant reduction when going

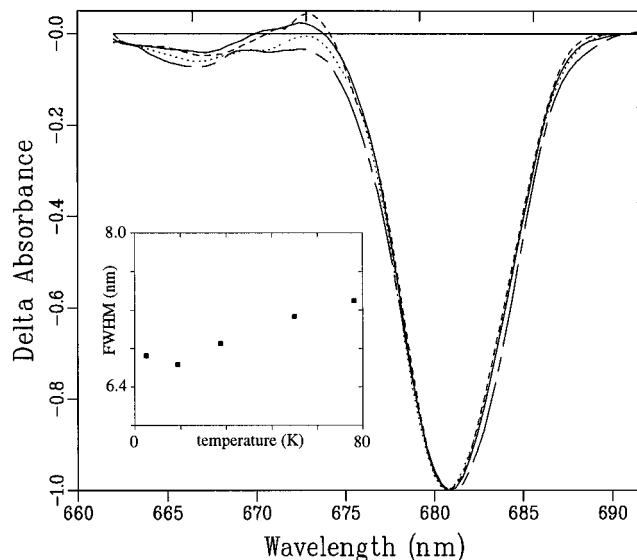


FIGURE 9: Triplet-minus-singlet absorbance difference spectra of RC_{DM-3} measured at $T = 4$ K (solid line), 15 K (dashed line), 30 K (dotted line) and 56 K (chain-dashed line). The inset shows the full width at half-maximum (FWHM) of the main bleaching as a function of temperature.

from 4.2 to 30 K (Figure 9, inset). Such a reduction is expected when the temperature rise results in the detrapping of red antenna chlorophylls by P-680 and thus in the disappearance of triplet states of these chlorophylls, and when these triplet states are red-shifted compared to the triplet state of P-680. Our results suggest therefore that the trap chlorophylls in the highly purified PS II RC preparations are not considerably red-shifted compared to P-680.

DISCUSSION

Purity of PS II RC Preparations. The results obtained by the gel filtration/diode array detection technique presented in this contribution conclusively show that PS II RC preparations binding about 6–6.5 Chl per 2 Pheo molecules can be obtained without any CP47 or other contamination. Not only was the PS II RC preparation obtained after a short treatment of CP47-RC complexes with Triton X-100 (Kwa et al., 1994a,b; Groot et al., 1994) found to be very pure (Figure 3) and binding 6.4 Chl per 2.0 Pheo (Eijkelhoff & Dekker, 1995), but also the PS II RC complex obtained without the help of Triton X-100 was found to be free of significant contaminations (Figure 5) and binding 6.5 Chl per 2.0 Pheo (Table 1).

The combination of the gel filtration technique and diode array detection appeared very sensitive for detecting CP47 or other contaminations. In Figure 5 it is shown that after two of the three purification steps the PS II RC complex isolated without the help of Triton X-100 (RC_{DM-2}) still contains a small amount of residual CP47: the A_{416}/A_{435} ratio drops from about 1.17 in the peak at 27–28 min to about 1.10–1.08 in the final tail of this peak at about 30–31 min, the time at which CP47 is expected to elute (Figure 1). A crude estimation of the amount of CP47 in the preparation reveals that it cannot be more than a few percent (on chlorophyll basis), because the total absorption at 30–31 min is very low and because at these elution times the PS II RC particles still dominate (the A_{416}/A_{435} ratio only drops to about 1.08, whereas for pure CP47 a ratio of ~ 0.75 is expected; see Figure 1). Such a contamination raises the number of

Chl per 2 Pheo by up to only 0.1 (2% contamination on a chlorophyll basis equals less than 1% contamination on a complex basis if 14 Chl's are assumed for CP47—Kwa et al., 1992b—and $0.99 \times 6.00 + 0.01 \times 14.00 = 6.08$), which is within the error limit of the pigment quantitation procedure (see also Table 1). We note that this very small contamination is removed in the final preparation (RC_{DM-3}): On the basis of Figure 5, we expect at least a 10-fold reduction of the CP47 contamination after the gel filtration purification step. This lowers the maximal CP47 contamination in RC_{DM-3} to 0.1%, from which we conclude that this preparation can be qualified as "ultrapure". We also note that the faint band in the immunoblot presented by Pueyo et al. (1995) corresponds to a much larger difference of 1–1.5 Chl per 2 Pheo molecules (corresponding to 10–20% CP47 contamination), from which we conclude that the gel filtration/diode array detection method is at least an order of magnitude more sensitive than the immunological method presented by Pueyo et al.

In Eijkelhoff and Dekker (1995) we discussed the possibility that the apparent lower number of Chl per 2 Pheo molecules in the preparations from the Picorel/Seibert group originates from their pigment quantitation method and proposed that in these preparations the actual number is also at least 6. The close resemblance between the absorption properties of the "4" Chl preparations from this group and our "6" Chl preparations is in agreement with this idea. The A_{416}/A_{435} ratio of the "4" Chl preparation reported by Pueyo et al. (1995) is within the error limit identical to that of RC_{short} (~ 1.20). In addition, the spectral fine structure of the 4 K absorption spectrum of this preparation (Chang et al., 1994a) with its deep valley between the 671 and 679 nm absorption bands shows a striking resemblance to that of RC_{DM} (Figure 7). These similarities contrast the differences that we recently observed (C. Eijkelhoff, F. Vacha, R. van Grondelle, J. P. Dekker, and J. Barber, submitted for publication) between our "6" Chl preparations and the "5" Chl preparations obtained by immobilized metal affinity chromatography (Vacha et al., 1995), which are characterized by clearly diminished absorption around 671 nm and an A_{416}/A_{435} ratio of at least 1.30, thus confirming the lower Chl content in this preparation.

With the gel filtration technique we were also able to detect a number of other possible contaminants. One of the most striking is the aggregated and almost pigment-free protein that was observed in RC_{long} (Figure 2). Since proteins other than those of the PS II RC complex were not observed by SDS PAGE (not shown), we interpret the presence of this type of protein as a result of Triton-induced denaturation and unfolding of the PS II RC complex during the anion-exchange step of the preparation of the complex. This interpretation also explains the low yield of the intact PS II RC complex that usually is observed upon long Triton washing. The possible presence of denatured proteins should also be taken into account in the discussions on the redox state of cytochrome *b*-559 (Shuvalov et al., 1994).

The gel filtration technique further enabled the observations of unbound pigments, and because free Chl *a* and free Pheo *a* migrate with slightly different velocities through the Superdex 200 column, it even allowed us to detect these pigments separately. For long Triton-treated preparations we found in several cases relatively large amounts of free Pheo *a* (see, e.g., Figure 2, where the free pigment peak at

32–33 min is dominated by Pheo *a* absorption—Pheo *a* is the only pigment of the PS II RC complex with an A_{416}/A_{435} ratio larger than 1), which suggests that at some stage during the isolation procedure free Chl *a* can be converted into free Pheo *a*. This implies that the pigment stoichiometry of newly isolated PS II RC preparations should be determined not only by HPLC or spectroscopic analysis of extracts in organic solvent but also by gel filtration in order to exclude the possibility that the presence of free Pheo *a* has disturbed the stoichiometry.

Properties of a Pure PS II RC Complex Isolated without Using Triton X-100. In this report it is described how PS II RC complexes can be purified without the use of Triton X-100. The major difference between this RC and those isolated with Triton appeared to be the β -Car content. This confirms the notion that the main effect of Triton X-100 is the extraction of β -Car from the complex (de las Rivas et al., 1993; Eijkelhoff & Dekker, 1995) and suggests that the "native" PS II RC complex binds two β -Car molecules, as was originally proposed by Kobayashi et al. (1990) and Gounaris et al. (1990). The gel filtration experiment has indicated that this isolated complex occurs in a single aggregation state, and it is most likely that the complexes are monomeric. This contrasts the results of Zheleva et al. (1995), who detected a mixture of monomeric and dimeric PS II RC complexes. This difference might originate from the different pretreatments that have been used. In particular, the treatment with *n*-octyl β -D-glucopyranoside (OGP) that we used to remove the major Chl *a/b*-binding complex LHC-II (Ghanotakis et al., 1987) may have induced a monomerization of the PS II complex.

Concerning the Chl content of this purified preparation, we again found contents between six and seven Chl for every two Pheo molecules. This could point to a heterogeneous population of particles, some binding six Chl's and some binding seven. It should not be ignored, however, that the error in the determination of the Chl content, which is an average of several extracts of several different preparations, is approximately 4% (~ 0.25 Chl) and that a possible small error in the extinction coefficient of Pheo *a* will result in an additional uncertainty in the estimation, due to which it cannot be excluded that the real Chl content is in fact a stoichiometric number of 6.0 or even 7.0 Chl per 2.0 Pheo.

The comparison of the red parts of the 4 K absorption spectra of RC_{DM-3} and RC_{short} by deconvolution into Gaussian components revealed only very minor differences, despite the much better resolved "valley" between the main bands at 671 and 679 nm in RC_{DM-3} and the differences in the red flank of the spectra. The most striking difference appears to be an increase by about 20% of the width of the two redmost bands in RC_{short}. This increased bandwidth might be attributed to an increase in the inhomogeneous disorder of the pigments responsible for the red-absorbing states in the Triton-derived preparation. Part of these red states have previously been ascribed to the multimer of P-680 (Durrant et al., 1995). 4 K spectra with deep valleys have also been reported in Triton-derived preparations (van Kan et al., 1990; Chang et al., 1994a; Cattanea et al., 1995), so it is not a rule of thumb that Triton-derived preparations show increased inhomogeneous disorder in the red absorption bands. In fact, we have now isolated RC_{short} preparations using a slightly different procedure that show the same degree of structure in the 4 K absorption spectrum as the RC_{DM-3} preparation.

Nature of the 684 nm Absorbing State. The results presented in Figure 8 of this contribution indicate that also the highly purified PS II RC complex contains one or more antenna molecules that trap excitation energy at very low temperatures and thus confirm earlier observations by Groot et al. (1994) on RC_{short} complexes. The trap consists primarily of chlorophyll, but it may contain some pheophytin contribution as well (Kwa et al., 1994b). It is characterized by a Gaussian distribution centered at 681.8 nm (Groot et al., 1996), which is about 1 nm blue-shifted compared to the peak of the red component in the 4 K absorption spectrum (Figure 7).

It was suggested by Chang et al. (1995) that the red trapping Chl is a special accessory Chl which acts as a linker between the CP47 core antenna and the RC and which could in principle be washed away with Triton X-100. These authors also proposed that the red trapping Chl would be responsible for the 684 nm shoulder in the 4 K absorption spectrum. If this were true, one would expect to observe less red trapping chlorophylls in preparations with less absorption in the 684 nm shoulder. The experiments with RC_{long} (the preparation with decreased absorption in the red shoulder) resulted, however, in a higher fluorescence yield at low temperatures (Figure 8, squares) and therefore suggest a higher contribution of the red trapping chlorophylls, which is just opposite to what was expected on the basis of the prediction of Chang et al. Our experiments indicate that upon long Triton treatment the average energy level of the primary electron donor is blue-shifted compared to that of the trap, which agrees with the interpretation that at least part of the 684 nm shoulder should be attributed to P (Otte et al., 1992; van der Vos et al., 1992; Kwa et al., 1994a).

The triplet-minus-singlet absorbance difference measurements on RC_{DM-3} suggested no significant reduction of the width of the main bleaching upon increasing the temperature from 4K to 30K (Figure 9), which contrasts the earlier observations of Chang et al. (1995). There are two possible explanations for the differences between these results. First, the preparations of Chang et al. (1995) could have been contaminated with CP47, which also contains 684 nm absorbing states (Chang et al., 1994b; Groot et al., 1995). We note that no evidence for the absence of CP47 has been presented in this study, that the preparations were apparently selected on the basis of a large 684 nm contribution, and that the CP47 triplet states shift to shorter wavelengths upon increasing the temperature (Groot et al., 1995), in agreement with the experimental finding of Chang et al. (1995). Second, it is possible that the preparations analyzed by Chang et al. (1995) resemble RC_{long} in the sense that they have a diminished P-684 contribution and therefore a blue-shifted distribution of the primary electron donor. In such preparations a relatively large contribution of the triplet state of trap is expected at 4 K, the intensity of which will indeed decrease upon raising the temperature, essentially following the fluorescence decrease. In the more intact preparations, the T-S difference spectrum is not expected to reveal a considerably narrower bandwidth upon raising the temperature, because the contribution of the triplet of the trapping pigments is relatively small and because the contribution of the P-684 triplet state will prevent the narrowing.

We conclude that the red part of the 4 K absorption spectrum is rather complex, even in the highly purified preparations, because of a heterogeneous population of the

primary electron donor and a significant contribution of the trap pigments. The trap itself may be heterogeneous as well, in view of the increasing evidence of the participation of both chlorophyll and pheophytin in this fraction. A detailed thermodynamic description of the complete pigment system such as the one presented by Groot et al. (1994) is therefore absolutely essential to estimate the relative contributions of the several triplet states.

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